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By Nina Salamah

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Total alkaloid content in various fractions of *Tabernaemontana sphaerocarpa* Bl. (Jembirit) leaves

N Salamah, D S Ningsih

Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia

E-mail: ninasalamah1996@gmail.com

Abstract. *Tabernaemontana sphaerocarpa* Bl. (Jembirit) is one of the *Apocynaceae* family plants containing alkaloid compound. Traditionally, it is used as an anti-inflammatory medicine. It is found to have a new bisindole alkaloid compound that shows a potent cytotoxic activity in human cancer. This study aimed to know the total alkaloid content in some fractions of ethanolic extract of *T. sphaerocarpa* Bl. leaf powder was extracted by maceration method in 70% ethanol solvent. Then, the extract was fractionated in a separatory funnel using water, ethyl acetate, and hexane. The total alkaloid content in each fraction was analyzed with visible spectrophotometric methods based on the reaction with Bromocresol Green (BCG). The total alkaloids in water fraction and ethyl acetate fraction were $(0.0312 \pm 0.0009)\%$ and $(0.0281 \pm 0.0014)\%$, respectively. Meanwhile, the total alkaloid content in hexane was not detected. The statistical analysis, performed in SPSS, resulted in a significant difference between the total alkaloids in water fraction and ethyl acetate fraction. The total alkaloid in water fraction of *T. sphaerocarpa* Bl. was higher than the one in ethyl acetate fraction.

Keywords: *T. sphaerocarpa* Bl., alkaloid, visible spectrophotometry, fractionation

1. Introduction

Alkaloid is a secondary metabolite compound that has been providing more benefits to the world of medicine and pharmaceutical dosage form compared to the other metabolites. As a type of secondary metabolites, it shows a wide biological activity. It occurs in plants, fungi, bacteria, amphibians, insects, marine animals, and humans. Plants and fungi, which are rich in natural materials, were used as pain relief in ancient times [1]. The *Tabernaemontana* species from the *Apocynaceae* family particularly has copious indole alkaloids. This type of alkaloid is an indicator compound that offers a wide range of benefits and plays an immense role in certain species classification [2].

9 The research on *T. sphaerocarpa* Bl stem [3] found two new bisindole alkaloid compounds, namely biscarpamontamine A, having an aspidosperma-iboga-type skeleton, and biscarpamontamine B, possessing an aspidosperma-aspidosmema-type skeleton. Biscarpamontamine A shows potent cytotoxicity against various types of human cancer cells.

The isolation of alkaloids from plant sources needs a good solvent to extract them, i.e., organic solvents such as ether, alcohol, and benzene. Fractionation is a further isolation of chemical constituents from the crude extracts to obtain purer compounds and hence, accentuate their influence on a specified bioassay test [4]. The major chemical property of alkaloids is their basic nature, which



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causes them to easily decompose following exposure to heat and light in the presence of oxygen. Physically, alkaloids are colorless. The free alkaloidal base is soluble only in an organic solvent, whereas alkaloidal salts are highly soluble in water [5].

A preliminary detection of alkaloids is achievable using Mayer's and Dragendorff's reagents. Mayer's reagent contains potassium iodide and mercuric chloride, while Dragendorff's reagent consists of bismuth nitrate and potassium iodide in a solution of nitric acid [6]. Alkaloids react with Mayer's reagent and produce cream-colored precipitate. Furthermore, Dragendorff's reagent detects a positive presence of alkaloid from the formation of brown to orange precipitate and the absence of such precipitate when added with H_2SO_4 [7].

The determination of total alkaloids using a visible spectrophotometric method with Bromocresol Green (BCG) is a simple and sensitive technique that requires no special equipment. BCG can react with certain alkaloids, i.e., the ones that have nitrogen inside their structure, but not with amine and amide alkaloids. The reaction of alkaloids with BCG forms a yellow-colored product [8].

Based on the previous research on the effects of extraction methods on the number of total alkaloids in *T. sphaerocarpa* Bl leaf, maceration produces higher total alkaloids and is thereby a better extraction technique than Soxhlet extraction [7]. Fractionation is a method used to separate one component from the others or to break a mixture into its constituent parts. The fractionation of a component can be carried out using chromatography. The isolation of alkaloids from plant sources needs a good solvent to extract them, i.e., organic solvents such as ether, alcohol, and benzene [4]. Alkaloids are separated from crude drugs using an extraction technique with non-aqueous organic solvent. Most alkaloids are present in plants in the form of salts soluble in methanol or ethanol [9].

2. Materials and Methods

2.1. The determination of *T. sphaerocarpa* Bl. leaf

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The determination took place in the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Ahmad Dahlan, Yogyakarta using *T. sphaerocarpa* Bl. leaf.

2.2. Sample processing

The collected *T. sphaerocarpa* Bl. leaves were washed, and then dried in an oven at a temperature of 60°C. After drying, the leaves were powdered using a grinder and passed through a multi-stage sieve to obtain 50/70 mesh particle size.

2.3. Extract preparation

The extract was prepared using maceration with three replications. Each extraction cycle used 150 gram of 50/70-mesh powder of *T. sphaerocarpa* Bl. leaf that was macerated with 500 mL of 70 % v/v ethanol. This 24-hour maceration required a 3-hour stirring first. It was repeated three times until the alkaloids were extracted perfectly. The decision of a perfect extraction was based on a qualitative observation on the macerate. Furthermore, the macerate was filtered with Buechner funnel under vacuum. The remaining solvents in it were removed using a rotary evaporator to obtain a viscous extract, which was tested qualitatively using Mayer's and Dragendorff's reagents. A positive reaction of alkaloids with Dragendorff's reagent is shown by the presence of orange-colored precipitate. Meanwhile, the presence of alkaloids is evidenced by the resultant cream-colored precipitate.

2.4. Non-specific standardization

2.4.1. Chemical yield calculation

The chemical yield was obtained by measuring the final weight (viscous extract) and comparing it with the initial weight of the powder before extraction.

2.4.2. Loss on powder drying and extract's water content

The loss on drying and the moisture content of the extract were determined with Halogen Moisture Analyzer. This instrument was positioned horizontally by adjusting the leveling screw. It is perfectly horizontal when the air bubble in the water pass is in the center. Then, the Moisture Analyzer Balance was switched on and left for 30 minutes before using. The red button was pressed until the display was on. After the start button on the display was pressed, the heating module was opened to load the pan. It was then closed, and the zero button was pressed to set the Moisture Analyzer to zero. Afterward, the

heating module was opened again, a sample of at least 0.5 grams of *T. sphaerocarpa* Bl. leaf extract was placed on the pan, and the heating module of the moisture analyzer was closed.

2.5. Qualitative analysis of alkaloids

The extract was dissolved in 3.0 mL of 70% v/v ethanol and added with 5.0 mL of HCl 2 M and 0.5 g of NaCl. This filtrate was then filtered, added with 3 drops of HCl 2 M, and divided into 4 tubes. Filtrate A functioned as a blank solution. Meanwhile, filtrate B was added with Mayer's reagent, filtrate C was added with dilute H₂SO₄, and filtrate D was added with Dragendorff's reagent [10].

2.6. Fractionation and the determination of total alkaloids

Five grams of the viscous extract were dissolved in 10 mL of water. Sonication was performed and a little of ethanol was added only when necessary (to help the dissolution process). This solution was then poured into a separating funnel, added with 10 mL of hexane, and shaken to produce a solution with two phases, namely water and hexane. These two phases were separated and collected. The extraction with hexane solvent was carried out until the hexane phase had the same color as the hexane solvent. The water phase was collected for fractionation using 10 mL of ethyl acetate. The fractionation was repeated until the ethyl acetate phase has the same color as the original ethyl acetate solution. The total alkaloids in every fraction, namely water, ethyl acetate, and hexane, were analyzed with UV-Vis spectrophotometric method.

2.6.1. The determination of operating time

A strychnine solution with a concentration of 25 µg/mL was poured into a separating funnel, added with 5.0 mL of phosphate buffer solution at pH 4.7 and 5.0 mL of BCG solution, and extracted with 5.0 mL of chloroform (two times). The chloroform phase was then taken. The extraction results were collected in a 10.0 mL volumetric flask and added with chloroform up to the mark. Then, the stable absorption time was checked at a wavelength of 470 nm. The data obtained from this analysis was used to make a curve representing the relationship between absorbance and time.

2.6.2. The determination of wavelength at maximum absorbance

A strychnine solution with a concentration of 50 µg/mL was poured into a separating funnel, added with 5.0 mL of phosphate buffer solution at pH 4.7 and 5.0 mL of BCG solution, and extracted with 5.0 mL of chloroform (two times). The chloroform phase was then taken. The extraction results were collected in a 10.0 mL volumetric flask and added with chloroform up to the mark. The absorbance of this solution was then checked at a wavelength of 350-700 nm using the operating time obtained from the above analysis. The resultant data in this analysis was used to make a curve representing the relationship between absorbance and wavelength.

2.6.3. The determination of standard curve

A standard strychnine solution with a concentration of 50 µg/mL was pipetted out by 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mL into a separating funnel, added with 5.0 mL of phosphate buffer at pH 4.7 and 5.0 mL of BCG solution, and extracted with 5.0 mL of chloroform (two times). The chloroform phase was then taken. The extraction results were collected in a 10.0 mL volumetric flask and added with chloroform up to the mark. Afterward, their absorbance was checked against the maximum wavelength.

2.6.4. Sample preparation

The water and ethyl acetate fractions, whose solvents had been evaporated, were added with 2N HCl until dissolved perfectly. Then, 1.0 mL of filtrate was extracted using 5.0 mL of chloroform (three times). Afterward, the pH of the solution was neutralized using 0.1N NaOH and added with 5.0 mL of phosphate buffer solution at pH 4.7 and 5.0 mL of BCG solution. This mixture was then extracted again using 5.0 mL of chloroform (two times). The chloroform phase was collected in a 10.0 mL volumetric flask and added with chloroform up to the mark. The absorbance at the maximum wavelength was considered in the calculation of the total alkaloid content.

3. Results and Discussion

The determination of stem, leaf, and stalk took place in the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Ahmad Dahlan, Yogyakarta. The results proved that

the plant used in this research was jembirit. It is *T. sphaerocarpa* Bl. species from the family *Apocynaceae* and the genus *Tabernaemontana*.

The water content of the powder of *T. sphaerocarpa* Bl. leaf in the three replications was 4.72%, 4.31%, and 8.32%, respectively. The data is presented in details in Table 1.

Table 1. The weight loss of *T. sphaerocarpa* Bl. leaf powder in drying

Replications	Weight of powder (gr)	Loss on Drying (%)	Mean \pm LE (%)	CV (%)
1	0.508	4.72	4.56 \pm 0.544	4.809
2	0.511	4.31		
3	0.510	4.65		

It shows that the weight loss of the powder of *T. sphaerocarpa* Bl. leaf in drying meets the requisites of Indonesian Herbal Pharmacopoeia (IHP) i.e., less than 10%. This percentage ¹⁰ assures that the powder can be stored and preserved from molds and other fungi. The chemical yield of the ethanolic extract of *T. sphaerocarpa* Bl. leaf is presented in Table 2.

Table 2. The chemical yield of the ethanolic extract of *T. sphaerocarpa* Bl. leaf

Replications	Crude drug (g)	Weight of extract (g)	Chemical Yield (%)	Mean \pm LE (%)
1	150.09	34.8	23.186	22.364 \pm 2.154%
2	150.12	33.7	22.448	
3	150.06	32.2	21.458	

Chemical yields signify the effectiveness of an extraction process in an analysis. The effectiveness is influenced by various factors, namely extraction solvent, stirring speed, the particle size of the powder, and the length of the extraction process. The calculation results showed that the mean of the chemical yield was 22.364%.

The moisture content was measured by the amount of water found in the material. In this research, it was measured to provide the maximum limit or range of the water content in the material [11]. The water content of the extract is summarized in Table 3.

Table 3. The determination of the water content in the ethanolic extract of *T. sphaerocarpa* Bl. leaf

Replications	Weight of powder (g)	Loss on Drying (%)	Mean \pm LE (%)	CV (%)
1	0.508	10.63	10.203 \pm 0.956%	3.773
2	0.506	10.10		
3	0.516	9.88		

Materia Medika Indonesia (MMI) explains that the water content of a sample is never less than 10% unless stated otherwise. A viscous extract containing 30% water is susceptible to the growth of microorganisms [12]. The water content in a pharmaceutical dosage form largely depends on extraction solvent, evaporation time, and storage conditions. In this research, the water content exceeded the upper limit of the allowed presence set by MMI possibly because of the solvent used in the extraction and the loosely sealed container that stored the extract.

The qualitative analysis of the ethanolic extract of *T. sphaerocarpa* Bl. leaf resulted in the presence of alkaloids. The positive result was evidenced by the cream-colored precipitate, i.e., from the reaction of alkaloids with Mayer's reagent, and the brick-red one, i.e., the response of alkaloids to Dragendorff's reagent. The whitish precipitate from the usage of Mayer's reagent is a potassium-alkaloid complex.

The determination of total alkaloids using a visible spectrophotometric method with Bromocresol Green (BCG) is a simple and sensitive technique that requires no special equipment. BCG can react

with certain alkaloids, i.e., the ones that have nitrogen inside their structure, but not with amine and amide alkaloids. The reaction of alkaloids with BCG forms a yellow-colored product, as presented in figure 1[8].

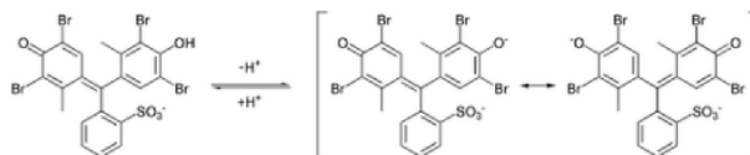


Figure 1. The structure and resonance of bromocresol green

The determination of the operating time (OT) of the samples was performed because the exact alkaloid content in *T. sphaerocarpa* Bl. was unknown. The results showed that the standard OT was 34-37 minutes, while the OT of the samples was 37-39 minutes. Furthermore, they also revealed that the maximum wavelength of the BCG complex with strychnine was 416 nm, while that of the BCG complex with alkaloids in *T. sphaerocarpa* Bl. leaf was 412.8 nm. The curve for the standard strychnine was measured at a maximum wavelength of 416 nm and within an operating time of 43-47 minutes. The absorbance obtained from the series of the standard strychnine solution is presented in Table 4.

Table 4. The absorbance of standard strychnine in different concentrations

Concentrations ($\mu\text{g/mL}$)	Absorbance
5	0.276
10	0.348
15	0.446
20	0.502
25	0.638
30	0.662
35	0.770

The equation obtained from the standard curve is $y=0.1644x+0.1914$ where x is content ($\mu\text{g/mL}$), and y is absorbance. The r -value, calculated from the standard curve, is 0.9941. The quantitative analysis using spectrophotometry provided a dataset of the absorbance of each sample, as presented in Table 5. The absorbance levels of the hexane fraction samples were 0.005, 0.062, and 0.016. Because these samples had a low absorbance, the quantification of the alkaloid content in these samples was not performed. The calculation results, as seen in Table 5, showed that the total alkaloid content in water fraction was higher than the one in ethyl acetate fraction.

Table 5. The calculation results of the determination of total alkaloid content in *T. sphaerocarpa* Bl. leaf

Fractions	Replications	Sample weights (g)	Absorbance	Total Alkaloid Content (%)	$X \pm \text{LE} (\%)$	CV (%)
Water	1	4.9984	0.700	0.0310	0.0312 \pm 0.0009	1.2820
	2	5.0037	0.711	0.0317		
	3	5.0141	0.702	0.0310		
Ethyl acetate	1	4.9984	0.310	0.0289	0.0281 \pm 0.0014	2.1352
	2	5.0037	0.306	0.0279		
	3	5.041	0.305	0.0276		

4. Conclusions

The total alkaloid content in water fraction was $(0.0312 \pm 0.0009)\%$, while the total alkaloid in ethyl acetate fraction was $(0.0281 \pm 0.0014)\%$. However, the absorbance of alkaloids in hexane fraction was not detected by the visible spectrophotometric method. The fractionation using water solvent contained higher total alkaloids than using ethyl acetate.

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